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Control of multidendritic neuron differentiation in *Drosophila*: The role of Collier

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Abstract

Proper sampling of sensory inputs critically depends upon neuron morphogenesis and expression of sensory channels. The highly stereotyped organisation of the *Drosophila* peripheral nervous system (PNS) provides a model to study neuronal determination and morphogenesis. Here, we report that Collier/Knot (Col/Kn), the *Drosophila* member of the COE family of transcription factors, is transiently expressed in the subset of multidendritic arborisation (da) sensory neurons that display an highly branched dendritic arborisation, class IV neurons. When lacking Col activity, class IV da neurons are formed but display a reduced dendrite arborisation. Col control on dendrite branching is distinct from that exerted by Cut, another transcription factor expressed in class IV neurons and necessary for proper dendrite morphogenesis. Col is also required for the class IV da-specific expression of *pickpocket* (*ppk*), which encodes a degenerin/epithelial sodium channel subunit required for larval locomotion. Characterisation of the *col* upstream region identified a 9-kb *cis*-regulatory region driving *col* expression in all class IV md neurons, even though these originate from two types of sensory precursor cells. Altogether, these findings indicate that *col* is required in at least two distinct programs that control the morphological and sensory specificity of *Drosophila* md neurons.

Keywords: *Drosophila*; Multidendritic neurons; Collier/knot transcription factors; PNS

Introduction

Results from the past 10 years have now clearly established that neuronal cell fate does not result from the action of a single regulatory gene, but rather from the combinatorial action of multiple transcriptional regulators. Given the enormous diversity of neuronal cell identities and the great number of regulatory genes in the genome, the decoding of neuronal cell fate specification is just beginning.

Drosophila is an attractive model system for investigating the cellular and molecular basis of neuronal determination and morphogenesis. The entire embryonic and larval peripheral nervous system (PNS) has been described at the level of individually identified cells and is organised in a stereotyped pattern (Bodmer et al., 1989; Campos-Ortega and Hartenstein, 1997; Jan and Jan, 1993; Orgogozo and Grueber, 2005). There are 45 sensory neurons per abdominal hemi-segment that fall into two classes of neurons called type I and type II (Fig. 1; Orgogozo and Grueber, 2005). Type I neurons are characterised by a single dendrite and are associated with accessory cells. They innervate two main types of sensory organs: the external sensory organs (es organs) and the chordotonal organs. In contrast, type II neurons possess several dendrites and have been named multidendritic neurons (md neurons), as most of them have profuse dendritic arborisation that ends freely under the epidermis. The md neurons are further subdivided into the dendritic arborisation (da) neurons, the tracheal dendrite (td) neurons and the bipolar dendrite (bd) neurons (Bodmer et al., 1987; Grueber et al., 2002). There are 16 da neurons in each hemi segment A2–A6, and they can be classified into four categories, class I–IV, in order of increasing arbor complexity (Grueber et al., 2002). Recent functional studies suggest the involvement of da neurons in thermo- and/or pain sensation and coordination of rhythmic locomotion (Ainsley et al., 2003; Hwang et al., 2007; Liu et al., 2003; Tracey et al., 2003; Xu et al., 2004). Da dendrite morphogenesis is regulated by both an

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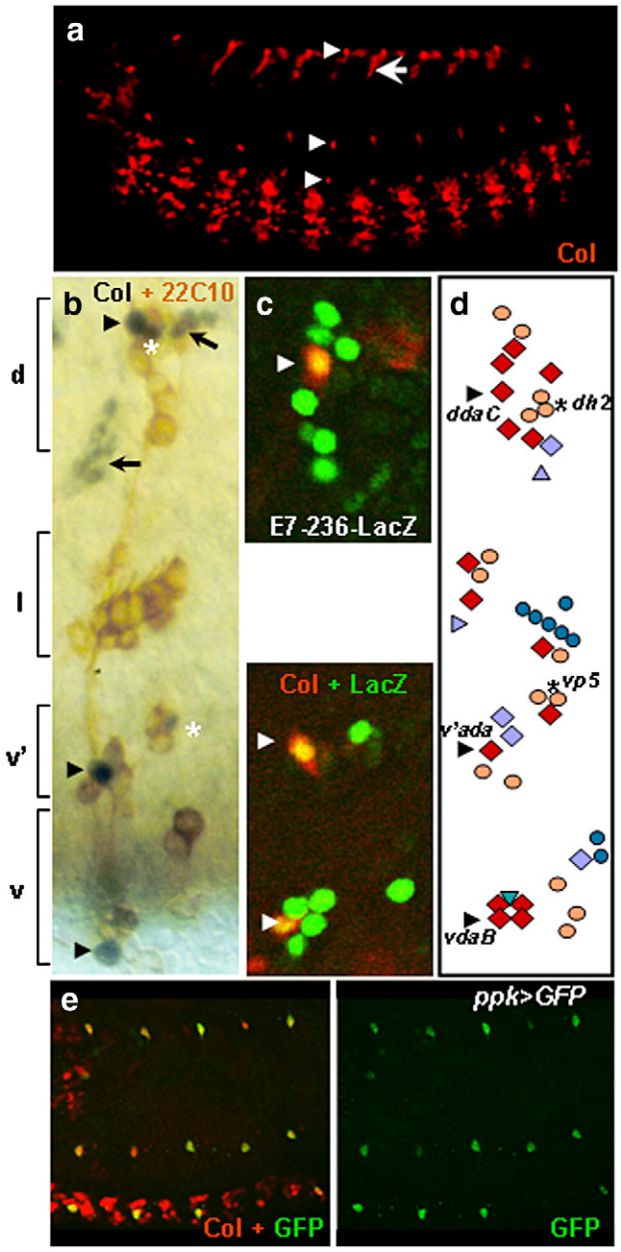


Fig. 1. Col expression in class IV md neurons. (a) Col expression in stage 15 embryos visualised by anti-Col antibody staining. Expression in PNS neurons and the DA3 somatic muscle is indicated by white arrowheads and a white arrow, respectively. Anterior is left and dorsal up in this and all subsequent panels. (b) Close-up view of and abdominal segment showing PNS neurons stained with 22C10 antibodies (brown) and *col* (black). The dorsal (d), lateral (l) and ventral clusters (v′ and v) are indicated. Black arrowheads and the black arrows point to the *col* expressing neurons and DA3 muscle, respectively. The white stars indicate two additional PNS neurons that express Col at a low level. (c) Detailed view of the dorsal (top) and ventral (down) clusters of md neurons in E7-2-36 embryos stained for Col (red) and LacZ (green). (d) Schematic representation of the *Drosophila* abdominal PNS modified from Orgogozo and Grueber (2005). da neurons, red diamonds; external sensory neurons, orange circles; other multidendritic neurons, purple diamonds and triangles; chordotonal organs, blue circles. The black arrowheads and black stars point to the ddaC, v'ada and vdaB md neurons and the dh2 and a neuron of the vp5 ES organ, respectively. (e) Overlapping expression of Col and *ppk*>GFP in abdominal segments from *ppk-Gal4/UAS*-GFP embryos stained with antibodies against GFP (green) and Col (red).

intrinsic program involving transcription factors expressed specifically in the neurons and an extrinsic program influenced by external factors such as peripheral glial cells (Yamamoto et al., 2006) or dendrite from similar neurons (Grueber and Jan, 2004). Numerous recent studies have identified an expanding number of genes that influence dendrite morphogenesis (Grueber et al., 2003a; Kim et al., 2006; Li et al., 2004; Parrish et al., 2006, 2007; Sugimura et al., 2004; Tassetto and Gao, 2006; Ye et al., 2007). Major questions now are (i) how do the regulatory proteins work together and (ii) what is the nature of the effector/target genes that generate neuron identity.

The COE (Collier/Olf-1/EBF) gene family contains a single member in all metazoan genomes except for vertebrates, where up to 4 genes have been found. *coe* genes have been shown to be expressed in subsets of sensory neurons in both cnidarians and various protostomes and deuterostomes (Dubois and Vincent, 2001; Dubois et al., 1998; Garel et al., 1997; Mazet et al., 2004; Pang et al., 2004; Prasad et al., 1998). The *Drosophila coe* gene *collier/knot* (designated below as *col*) is expressed in different embryonic tissues (Crozatier et al., 1996). Whereas *col* function in the development of a somatic muscle, the lymph gland and specific neurons of the central nervous system (CNS) have been described over the years (Baumgardt et al., 2007; Crozatier and Vincent, 1999; Crozatier et al., 1999, 2004), its function in PNS sensory neurons remained to be analysed.

Here, we report that *col* expression in the embryonic PNS is restricted to a subset of multidendritic neurons, the class IV md neurons. While class IV md neurons originate from two types of sensory precursors and are specified independently of Col, *col* is required cell autonomously, both for the larval development of the complex dendrite arborisation and the transcription of *pickpocket* (*ppk*), which encodes a degenerin/epithelial sodium channel subunit and is required for larval locomotion. *col* expression and function therefore provide a unique paradigm to investigate the transcriptional code that controls the sensory neuron identity within an evo–devo framework.

Materials and methods

Fly stocks

The following stocks were used: *ham*^{1} (Moore et al., 2002), *ppk*-Gal4 (Ainsley et al., 2003), *ppk*-GFP(Grueber et al., 2003b), UAS-mcd8GFP (Grueber et al., 2002), E7-2-36-LacZ, *Pcol85-Gal4* (Krzemien et al., 2007), *P9cG* (Dubois et al., 2007), *da*-Gal4, *elav*-Gal4 (DiAntonio et al., 2001) and Ig1-1, NP7028 and NP1156 (Sugimura et al., 2003). For MARCM analysis, we used the *elav*-gal4 UAS-mCD8-GFP, hs-FLP and the tub-Gal80, FRT42B strain (Bloomington Centre) and constructed the stock *y w*;FRT42B, *col1*/*CyO*. Generation of MARCM clones was performed as described by Grueber et al. (2003a). *col*-dsRNA experiments were performed at 29 °C.

Immunohistochemistry

In situ hybridization and immunostaining were performed as described (Crozatier et al., 1999). Antibodies used: mouse monoclonal α-Col (1/100; Dubois et al., 2007); mouse α-LacZ (1/800; promega); rabbit GFP (1/250; Torrey Pines Biolabs); mouse α-22C10 (1/20); rat α-elav (1/10); and mouse α-cut (1/100) are both from Developmental Studies Hybridomas Bank, Iowa City, US.

An antisens DIG-labelled RNA probe was performed for *ppk*. The clone dmdNAC1 (Darboux et al., 1998) is linearised by *Eco*RI, and T3 RNA polymerase is used for *in vitro* transcription.

Quantification

All pictures were acquired on a Leica TCS SP2 confocal microscope at ×20 magnification. For class IV md neurons, the total length of dendritic branches was measured using Metamorph software (Universal Imaging Corporation). The number of branching points was counted manually. Data are presented as means±SD. All statistical analyses were performed using Student's *t*-test.

To assess the relative level of Cut expression in *col* mutant compared to wt embryos, we used one cell-deep stacks of confocal images. Cut immunofluorescence was quantified in E7-2-36 embryos (to allow unambiguous identification of each neuron) double labelled with rabbit anti-Cut and mouse anti-βgalactosidase antibodies. Each neuron was manually outlined in Image J (NIH) to obtain the average pixel intensity for Cut and LacZ. To facilitate the comparison, the expression level was calculated as a ratio of the mean pixel intensity between Cut and LacZ for vdaB neurons ($n>20$ neurons). We found no significant difference between the control and *col* mutant (Student's *t*-test, confidence level of 0.8).

Results

Col expression in class IV multidendritic neurons

While studying the function of Col in the formation of the embryonic dorsal DA3 muscle, we observed that it was expressed in a specific subset of md neurons in the PNS (Crozatier and Vincent, 1999; Orgogozo and Schweisguth, 2004; Fig. 1a). *Drosophila* embryos display a stereotypical pattern of PNS organs – neurons and associated cells – that is highly reproducible. There are three types of PNS neurons: external sensory (es) organ neurons, chordotonal organs neurons and md neurons. The latter are organised in four clusters along the dorso-ventral axis of each abdominal hemisegment: dorsal (d), lateral (l), ventro-lateral (v′) and ventral (v) clusters, diagrammatically represented in Fig. 1d. Double immunostaining of embryos with anti-Col and 22C10, which recognizes neuron cell bodies and axons, indicated that Col is expressed in one neuron of each v, v′ and d cluster in the A1–A7 abdominal segments (Figs. 1b and d). Only two neurons, located in the dorsal and ventral clusters, express Col in the T1–T3 thoracic segments (Fig. 1a). Double Col/B-galactosidase immunostaining of embryos from the *E7-2-36* enhancer-trap line that expresses Lac-Z in all md neurons (Brewster and Bodmer, 1995) indicated that the PNS neurons expressing Col at stage 14 are, from ventral to dorsal, vmd1a/vdaB (Grueber et al., 2002), vmd4a/vdaa/v'ada and ddaC neurons (Fig. 1c, see also Orgogozo et al., 2002). Two additional Col-positive neurons per segment were also observed, at stage 15 (Fig. 1b). They correspond to dorsally one neuron innervating the dh2 es organ and ventrally one neuron innervating the vp5 es. According to (Brewster and Bodmer, 1995), the vp5 and dh2 neurons are late-born neurons, correlating with their late detection with Col antibodies. The precise lineages of the vp5 and dh2 neurons have not, however, been established (V. Orgogozo, personal communication). The vdaB, v'ada and ddaC neurons have been shown to correspond to the class IV md neurons, i.e., neurons displaying an extensive dendritic arborisation. They were also shown to specifically express *pickpocket* (*ppk*), a member of the family of degenerin/epithelial sodium channel subunits (Adams et al., 1998; Darboux et al., 1998; Grueber et al., 2002; Hattori et al., 2007; Orgogozo and Grueber, 2005). Double immunostaining of stage 16 *ppk*-Gal4-UASmcd8-GFP embryos with anti-Col and anti-GFP antibodies confirmed a complete overlap between both patterns (Fig. 1e). *ppk* transcripts were not detected, however, prior to stage 15, while *col* transcripts were first detected at late stage 11 in cells that we assume, based on their positions, are the precursor cells of md neurons, pIIb (see below, not shown). However, *col* transcription is no longer detected in the larval PNS (data not shown) while *ppk* remains expressed. Therefore, while perfectly overlapping at stage 15, and strictly specific of class IV md neurons, expression of *col* and *ppk* is temporally uncoupled.

Detailed studies performed over the years by many laboratories (Bodmer et al., 1989; Brewster and Bodmer, 1995; Orgogozo et al., 2001, 2002; Vervoort et al., 1997) have shown that the 22 md neurons present in each abdominal hemisegment originate from at least two distinct lineages, starting from sensory organ precursor (pI) cells (Lai and Orgogozo, 2004). In the "md-solo" lineage, pI divides asymmetrically twice but generates only one md neuron since the secondary precursors pIIa and pIIIb undergo apoptosis (Fig. 2; Orgogozo et al., 2002). The second lineage called "md-es lineage" involves four asymmetric cell divisions that generate five distinct cells, the four es organ cells and the md neuron (Fig. 2c; Orgogozo et al., 2001). The transcription factor Hamlet (Ham) is expressed in the es neuron but not in the md neuron and acts as a binary genetic switch between these two cell fates, with the loss of *ham* function converting the es neuron into an md neuron (Moore et al., 2002, 2004). In *ham* mutant, the md neurons originating from md-es lineages are duplicated whereas the vdaB neuron that originates from an md-solo lineage is not (Grueber et al., 2003b; Orgogozo et al., 2002). Staining of *ham* mutants embryos with anti-Col antibodies revealed only one dorsal and one ventral Col-expressing md neuron per abdominal hemisegment, as is the case in wild type, indicating that the ddaC and vdaB md neurons both originate from a "md-solo lineage" (Figs. 2a and b). By contrast, two rather than one Col-positive cells were frequently (around 80% of hemi-segments) observed at the position of the v'ada md neuron, confirming that the v'ada neuron is specified from an md-es precursor (Orgogozo et al., 2001). The same results were obtained using *ppk* as a probe (data not shown). Based on these data, we conclude that the three class IV md neurons originate from two different types of md lineages.

col is required for ppk expression but not for initial dendrite morphogenesis of class IV neurons in the embryo

Since *col* and *ppk* are specifically and sequentially transcribed in class IV md neurons, we tested whether *ppk* expression was dependent upon Col activity. We found that *ppk* expression was totally absent in *col* (col^1 null allele) mutant embryos (Figs. 3a and b), although transcription of *col* itself

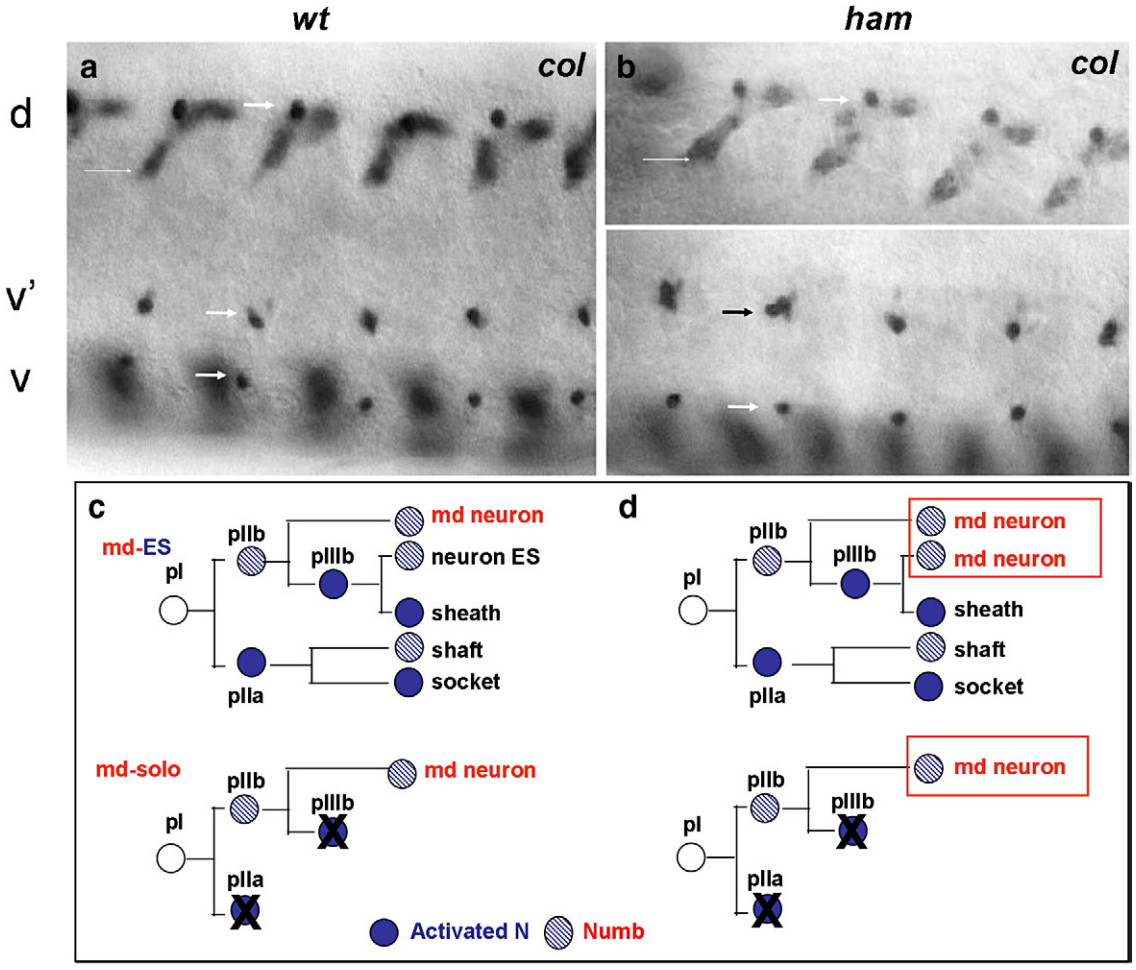


Fig. 2. Col-expressing class IV md neurons originate from two types of lineages. (a–b) Close-up view of abdominal segments of wt (a) and *ham* mutant (b) stage 15 embryos stained for Col. Positions of the Col expressing neurons in the dorsal (d) and ventral (v and v′) clusters are indicated by white arrows. The thin white arrow points to the DA3 Col expressing muscle. (b) Two Col-positive neurons (black arrows) are observed in the v′ cluster in *ham* mutants. (c–d) md-es (Orgogozo et al., 2001) and md-solo (Orgogozo et al., 2002) lineages are indicated in *wt* (c) and *ham* mutant embryos (d). Cells inheriting Numb (dashed blue) and cells where Notch signalling is activated (blue) are indicated. In *ham* mutants, only the md-es lineage gives rise to two md neurons (adapted from Orgogozo et al., 2002).

was maintained (data not shown), indicating that *col* expressing neurons are specified but do not activate *ppk* transcription. LacZ staining of *col* mutant embryos carrying the *E7-2-36* enhancer trap confirmed that Col-expressing neurons are specified as md neurons, independent of Col (Fig. 3c). Another md marker is the homeodomain protein Cut, which allow to distinguish between the different morphological types of md neurons (Grueber et al., 2002). Class III, IV, II and I md neurons express high, medium, low and undetectable levels of Cut, respectively and these Cut expression levels control dendrite branching patterns (Grueber et al., 2003a). One possibility was that Col could regulate Cut expression in class IV md neurons. We therefore compared Cut expression between wt and *col* mutant embryos, using the enhancer trap *E7-2-36*, to unambiguously identify md neurons. No difference in the level of Cut expression could be observed ($n>10$ in wt and col^1 mutants clusters examined; Fig. 3d and data not shown). This result indicates that *col* does not control the specific level of Cut in class IV md neurons, a conclusion also reached by Hattori et al., 2007. To examine the dendrite pattern of class IV neurons in *col* mutant embryos, we chose to express mcd8-GFP, a membrane-bound form of GFP, in class IV neurons. Since the *ppk* reporter constructs used by others to follow class IV neurons are, like *ppk* itself, not expressed in *col* mutant embryos (not shown), we mapped the *col* upstream region responsible for *col* expression in the class IV md neurons, using lacZ reporter constructs. We found that a reporter gene containing 9 kbp of *col* upstream DNA displayed a robust md expression (Fig. S1; Dubois et al., 2007). We subsequently constructed a corresponding Gal4 driver, P9cG. Class IV md neurons exhibit a complex and typical dendrite pattern, which starts to form at the end of embryogenesis (Figs. 3e and f; Sugimura et al., 2004). P9cG-driven expression of mcd8-GFP revealed no difference in this pattern between *wt* and *col* mutant embryos. Altogether, these data show that the transient embryonic *col* expression in class IV neurons is required for the specific expression of *ppk*, but not for the specification of the initial dendrite pattern.

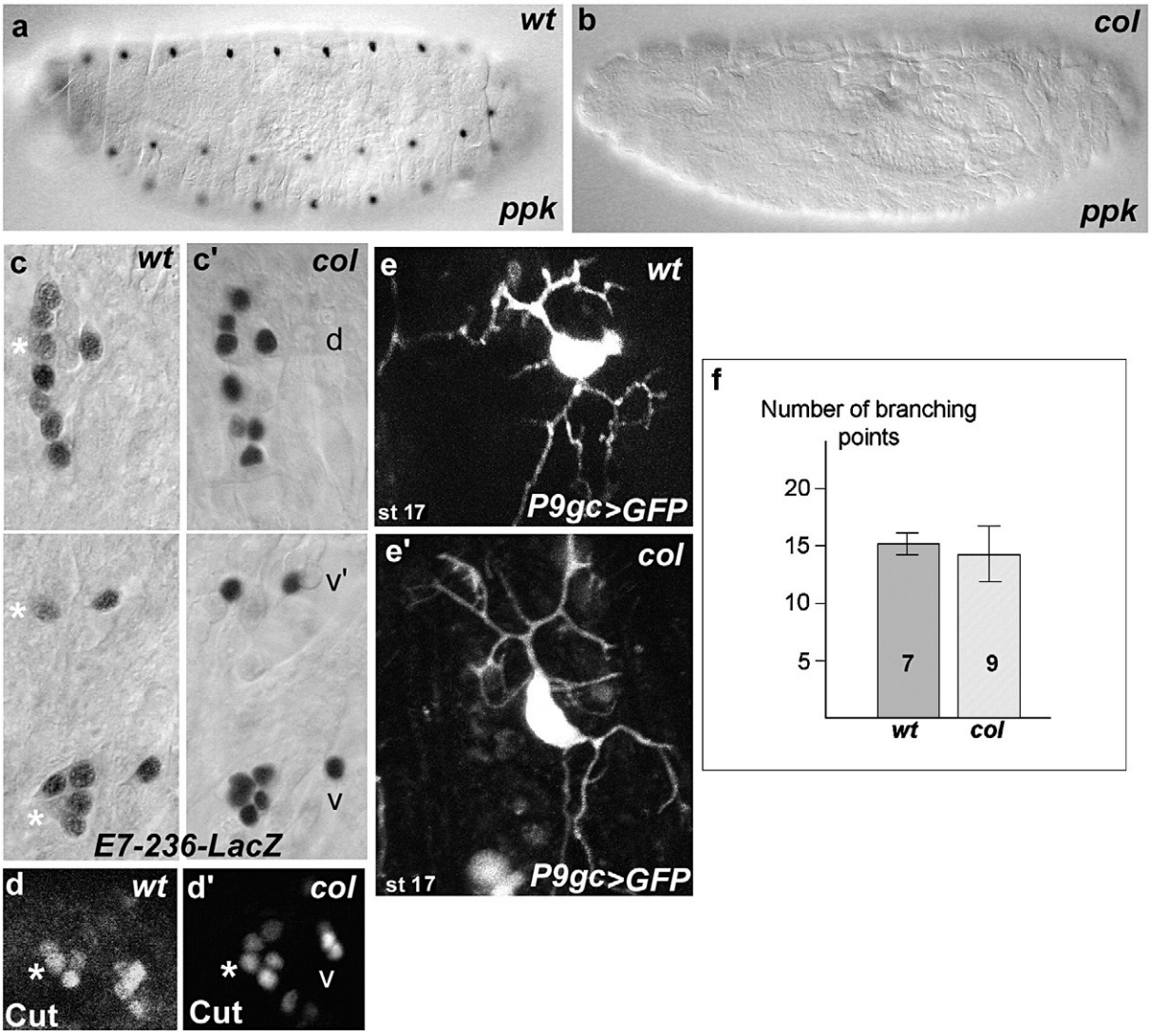


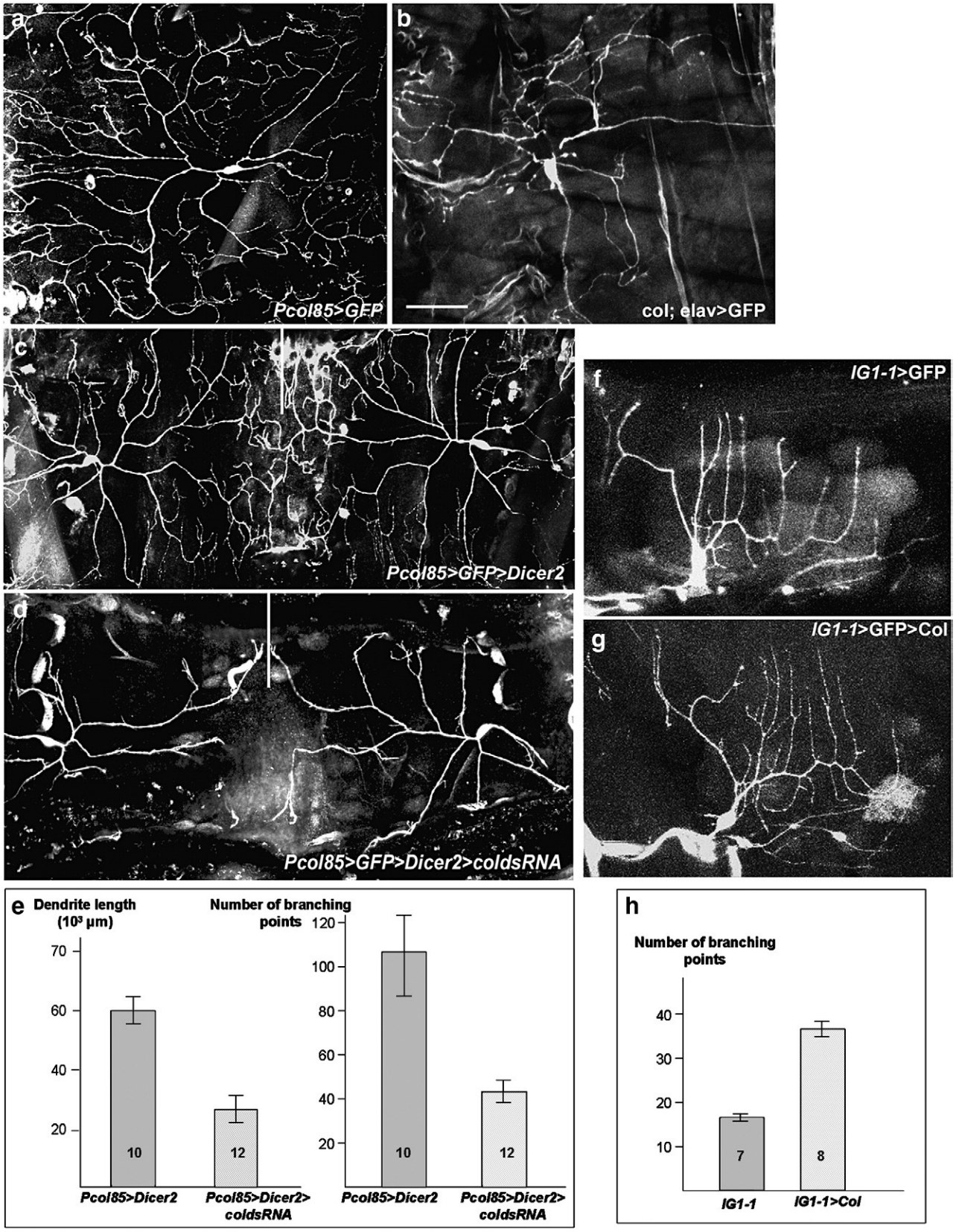
Fig. 3. Col controls *ppk* but not Cut expression. (a, b) *ppk* expression, as visualized by *in situ* hybridization in stage 16 embryos (a), is lost in *col* mutant embryos (b). (c–c′) Close-up view of abdominal segments of E7-2-36 embryos expressing LacZ in all md neurons. Lac Z immunostaining is identical in wt (c) and *col* mutant embryos (c′). The Col expressing md neurons in the d, v and v′ clusters are indicated by white stars. (d–d′) Detailed view of Cut expression in neurons of the ventral cluster md neurons. No difference in the level of Cut expression in the vdaB md neuron (white star) is observed between wt (d) and *col* mutant (d′) embryos. (e–e′) P9gc-driven expression of mCD8-GFP in a ddaC md neuron in stage 17. GFP labels the dendritic network that starts to develop. No difference can be observed between *wt* (e) and *col* mutant (e′) embryos. (f) Quantitative analysis of the number of branching points. The number of neurons analysed is indicated in each panel. No significant difference was found between control and *col* mutant neurons (Student's *t*-test, with a confidence level of 0.6).

col is required for higher-order dendrite branching of class IV neurons in larvae

The primary branches of class IV md neurons start to form at the end of embryogenesis and continue to elongate during larval development while, at the same time, a more elaborate dendrite network develops (Sugimura et al., 2003). In order to determine if *col* is required in the establishment of this complex branching network during larval development, we decided to look at the dendrite network of class IV md neurons

Fig. 4. Col is required for establishment of the secondary network of branching dendrites of class IV neurons. (a, b) Dendrite network of class IV md neurons visualised by expression of mcd8-GFP (Pcol85-Gal4 driver; GFP immunostaining) (a) wt v'ada neuron. (b) *col* mutant v'ada neuron obtained by MARCM. In both cases, the dendrites reach the segmental border and cover a large area of the epidermis, a characteristics of class IV md neurons. In *col* mutant neurons, the secondary dendrite branching is less developed than in *wt*. (c, d) *wt* ddaC neurons (c) or (d) neurons expressing *col-dsRNA* together with mcd8-GFP and Dicer2 under the control of Pcol85-Gal4. Dendrite branching is drastically reduced upon expression of *col-dsRNA*. The white vertical bar indicates the position of the dorsal midline. (e) Quantitative analysis of the total length of dendrites and total number of branching points, showing significant differences between the control and *col*-dsRNA experiment (Student's *t*-test, confident level of 0.9995). The number of neurons analysed is indicated in each panel. (f–h) The dendrite network of class I md neurons (dorsal cluster) visualised by GFP immunostaining (*IG1-1-Gal4/UAS-mcd8-GFP*); (f) *wt* neurons; (e) neurons expressing Col (*IG1-1-Gal4/UAS-Col*). (h) Quantitative analysis of the total number of branching points showing significant differences between the control and *col* ectopic expression experiment (Student's *t*-test, confident level of 0.9995). The number of neurons analysed is indicated in each panel. Scale bar 40 μm (a–d). Anterior and ventral are down and left, respectively.

in *col* mutant larvae. Since *col* mutant embryos die at the end of embryogenesis/beginning of larval stage (Crozatier et al., 1999), we turned to the MARCM system to generate *col* mutant md neurons expressing mcd8-GFP in an otherwise *wt* larva (Lee and Luo, 1999). We used the null col^1 mutation and found that *col* mutant md neurons do survive to the 3rd instar



larval stage and extend dendrite trees that reach the intersegmental border and cover about a third of the hemisegment, as wild-type class IV md neurons. However, the pattern of secondary arborisation seemed to be less complex than in *wt* (Figs. 4a and b; Hattori et al., 2007). However, there are several drawbacks in the use of the MARCM technique. First it does not allow a direct comparison between adjacent *wt* and mutant neurons, which makes it difficult to detect minor defects in the secondary/tertiary arborisation. Second, we never observed events where a class IV md neuron was accompanied by another GFP-positive, neuron. The ddac and vdaB lineages predict that both an md neuron and the sibling es neuron should be labelled together if mitotic recombination occurred during the pI to pIIa/pIIb mitosis or in earlier mitosis (Fig. 1b). We therefore concluded that our MARCM labelling of md neurons resulted from mitotic recombination events during the pIIb to pIIIb/md division. Accordingly, this raised the possibility that low levels of Col activity could still be present in the MARCM-labelled cells and could rescue some aspects of the *col* mutant phenotype. In order to circumvent these potential problems, we decided to interfere with *col* function in class IV md neurons by expressing a *col*-dsRNA (UAS-*col*-dsRNA). Recent studies performed in CNS neurons established that *col*-dsRNA can efficiently shutdown Col activity; its effect is enhanced by co-expression of Dicer-2 (UAS-Dicer2; Baumgardt et al., 2007). The ability of *col*-dsRNA to reproduce a *col* loss-of-function phenotype was further ascertained in the wing, where *col* is required for the specification of the central intervein domain and the L4 vein (Crozatier et al., 2002). For this, we used a driver that reproduces most of *col* expression, including in the wing and class IV md neurons (Fig. S3 and *Pcol85-Gal4* driver; Krzemien et al., 2007). In the wing, *col*-dsRNA mimics the *col* loss-of-function phenotype. In the PNS, *col*-dsRNA expression resulted in a clear, reproducible and significant reduction of both the total length of the dendrite network and number of branching points in L3 larvae (Figs. 4c, d and e). A similar reduction was observed upon expression of a dominant-negative form of Col (EnRCOE) made by substituting the Engrailed repressor domain for the transactivating domain of mouse EBF2/COE2, under control of the *col* regulatory

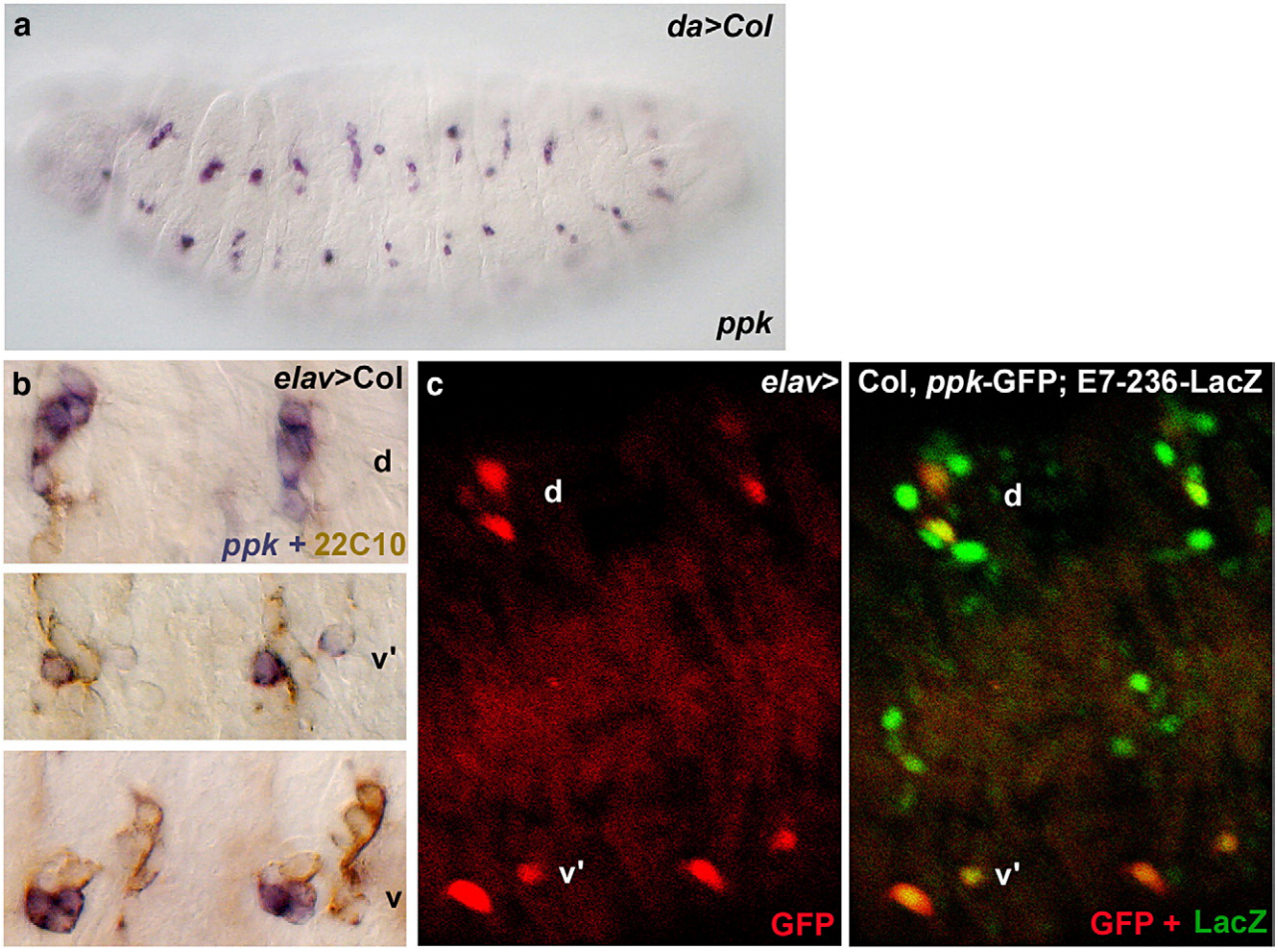


Fig. 5. Pan-neural Col expression induces ectopic *ppk* transcription in md neurons. *In situ* hybridisation showing ectopic activation of *ppk* in the PNS of stage 15 embryos upon ubiquitous expression of Col (*da-Gal4/UAS-col*). (b) Close-up view of the d, v and v′ clusters in a stage 15 embryo expressing Col in all neurons (*elav-Gal4/UAS-col*) visualised by 22C10 immunostaining (brown). Ectopic expression of *ppk* (black) is detected in md neurons. Two abdominal segments are shown. (c) Stage 15 embryo expressing Col in all neurons (*elav-Gal4/UAS-col*) and Lac-Z in md neurons (E7-2-36) and carrying a *ppk*-GFP reporter transgene. Immunostaining for GFP (left) or GFP and LacZ (right) shows an ectopic expression of *ppk* restricted to md neurons. Position of the d and v′ clusters is indicated.

sequences (Sébastien Mella, data not shown). Together with the MARCM results (Fig. 4; Hattori et al., 2007), it indicates that *col* activity in embryos is required for the elaboration of higher-order dendritic branching of class IV md neurons which takes place in larvae. To determine whether ectopic Col expression was able to induce changes in the dendrite network of md neurons other than class IV, we used the *IG1-1* diver (Sugimura et al., 2004) to express *col* from stage 15 embryo, specifically in class I md neurons. Class I md neurons display a dendrite morphology much simpler than class IV neurons. mcd8-GFP staining revealed significant increase in the number of branching points in dorsal class I in IG1-1>Col neurons compared to wild type (Figs. 4f–h), suggesting that *col* activity is able to modify the elaboration of the dendrite network of neurons other than class IV.

col acts in a context-dependent manner to activate ppk

To determine whether restriction of *ppk* expression to class IV neurons only depends on restricted Col expression or additional factors specific to these neurons, we looked at *ppk* transcription when Col was ubiquitously expressed throughout the embryo (*da-gal4* driver). We observed that *ppk* was indeed ectopically expressed in these conditions, but only in a small number of cells (Fig. 5a). The same restricted pattern of ectopic expression was observed when the pan-neural *elav-Gal4* driver was used, indicating that the ectopic *ppk*-expressing cells were probably neurons (Fig. 5b). Double immunostaining of *elav*-Gal4/UAS-Col;*ppk*-GFP;*E7-2-36* embryos for GFP and B-galactosidase further showed that all the *ppk*-positive cells are B-galactosidase-positive, indicating that the only PNS neurons competent to activate *ppk* expression in the presence of Col are md neurons (Fig. 5c). Like endogenous *ppk*, however, ectopic *ppk* expression was not detected prior to stage 15, i.e., several hours after the md neurons were born and had accumulated Col protein. Altogether, these data show that Col ability to activate *ppk* is both dependent upon (an) other md neuron-specific factor(s) and is limited to a specific time window, around embryonic stage 15.

Discussion

Each individual cell of the *Drosophila* embryonic PNS has been described, providing a unique model system to investigate the mechanisms of coding of neural identity (Bodmer et al., 1987; Campos-Ortega and Hartenstein, 1997; Jan and Jan, 1993; Orgogozo and Grueber, 2005). We show here that expression of the COE transcription factor Collier/Knot is specific to a subset of md neurons, the class IV neurons. Col is required both for the specific expression of *ppk*, a gene encoding a subunit of a sodium-gated channel involved in locomotion (Ainsley et al., 2003) and aspects of the elaborate dendritic branching pattern of class IV md neurons. It should be noted that an independent study of *col* function in md neurons came to very similar conclusions (Hattori et al., 2007).

Col expression in class IV md neurons: phenotypic convergence of two distinct md lineages

Systematic screens for transcription factors required for proper morphogenesis of *Drosophila* sensory neuron dendrites have revealed that a number of distinct transcription factors are involved in different aspects of dendrite extension, lateral branching and arborisation as well as in restriction of the dendritic coverage (Parrish et al., 2006). However, to date the expression pattern of very few of these transcription factors is known during embryonic and post embryonic development. To our knowledge, Col is the only known transcription factor whose expression is restricted to a subtype of md neurons. One particularly intriguing feature is that the three class IV md neurons found per abdominal hemisegment originate from at least two different types of lineages, the md-es (v'ada) or md-solo lineages (ddaC and vdaB), pointing to the existence of a convergence process towards the same neuronal phenotype. Since *col* remains transcribed in these three neurons in embryos mutant for *col*, it suggests that they are specified independently of *col* activity. The regulation of Col expression and the function in class IV md neurons provide a unique paradigm to study how the transcriptional control of md specification operates in independent lineages.

Temporal regulation of col expression in the class IV md lineages

col transcription is first observed in the pII cells at the origin of class IV md neurons, indicating that it becomes restricted to these neurons following asymmetric division of the pIIb cell (data not shown). Two or three Col-positive cells in place of each dorsal md neuron were often detected in embryos mutant for *sanpodo*, which encodes a four-pass transmembrane protein which interacts with the Notch receptor (data not shown and O'Connor-Giles and Skeath, 2003). Conversely, md-specific *col* transcription is often lost in embryos mutant for *numb* (*numb*^{1}, a null allele), which down regulates Notch signalling in one of the pIIb daughter cells (Crozatier and Vincent, 1999). We therefore conclude that *col* transcription is repressed by Notch signalling. Our previous studies on *col* requirement for the formation of the DA3 embryonic muscle showed that *col* transcription in the sibling DO5 muscle lineage is also repressed by Notch (Crozatier and Vincent, 1999). It thus appears that *col* repression by Notch is used in several independent cell lineage decisions. Whether the same effectors of the Notch pathway are involved both in the md neuronal and DA3 muscle lineages remains to be determined. One intriguing feature of *col* transcription in post-mitotic md neurons is that it is only transient. This could be due to a direct control by maternal and/or early zygotic transcription activators that dilute away during embryogenesis. Alternatively, it could involve the progressive accumulation in md neurons of a repressor able to shut down *col* transcription. Preliminary dissection of the *Drosophila melanogaster col* upstream region mapped the *cis*-regulatory information necessary for *col* transcription in md neurons to a fragment located between −9 and −5 kbp upstream of the

transcription start site (Dubois et al., 2007). Expression of the reporter gene was not detected, however, in the neurons innervating the dh2 and vp5 es organs, indicating that Col expression in md neurons and es neurons is under the control of separate *cis*-elements. Whether (partly) different *cis*-elements are also involved in md-solo, versus md-es *col* activation remains an open question. An identical pattern of Col expression in Class IV md neurons is observed in *Drosophila virilis*, indicating that the transcriptional regulatory network controlling this expression has been conserved between these two *Drosophila* species (Fig. S1). The −9- to −5-kbp fragment of *col* upstream DNA contains many sequences, between 20 and 80 bp in length, which are identical between *D. melanogaster* and *D. virilis* (Fig. S2) and represent as many potential regulatory sequences. Further dissection of this fragment should allow identifying which combinations of transcription factors are involved in the specific activation and temporal restriction of *col* transcription in class IV md neurons.

Control of ppk expression by col: a window of opportunity?

Activation of *ppk* transcription in class IV md neurons requires Col activity. Unlike Col, *ppk* remains expressed in these neurons until the end of larval development, showing that once activated, *ppk* expression is maintained independently of Col. This suggests the occurrence of a relay mechanism. The several hours delay between Col accumulation and *ppk* activation, in both normal embryos and upon pan-neuronal expression of Col, also indicates that the ability of Col to activate *ppk* depends upon another md-specific factor. Whether this factor(s) is itself a target of Col only activated in md neurons or an md-specific factor whose activity is potentiated by Col remains to be investigated. Finally, whether and how the delay and maintenance mechanisms are coupled is also unknown, at present. The transient character of Col expression does not favour a feed-forward mechanism such as that proposed for the specification of two lineage-related neurons in the CNS, where Col and its downstream targets act together to activate lineage-specific neuropeptides (Baumgardt et al., 2007). The *cis*-regulatory region driving *ppk* expression in class IV md neurons has been identified (Grueber et al., 2003b). Parallel studies on *col* and *ppk* *cis*-regulation should now allow deciphering more precisely the transcriptional control of class IV md neuron differentiation.

Two parallel programs regulate gene specific expression in class IV md neurons

In addition to *ppk* expression, class IV md neurons differ from the other md neurons by the length and degree of arborisation of their dendrite network (Grueber et al., 2002). This dendritic arborisation is unchanged in *ppk* mutant larvae (Ainsley et al., 2003 and W.A. Johnson, personal communication), showing that *ppk* expression and the dendritic network of class IV md neurons are specified by two independent programs. Formation of the primary branches of the md dendrite network starts to form at the end of embryogenesis and continues to elongate during larval development. At the same time, a more elaborate pattern of secondary arbors develops (Grueber et al., 2002; Sugimura et al., 2003). The recent identification of no less than 76 transcription factors influencing (class I, in that case) dendrite formation (Parrish et al., 2006; Tassetto and Gao, 2006) suggests that the formation and maintenance of the dendritic network is regulated at many different, likely successive levels. One of these factors is Cut, which regulates distinct dendrite branching patterns in different md classes in a dose-dependent manner (Ainsley et al., 2003; Grueber et al., 2003a; Hattori et al., 2007). We therefore propose that Col plays a dual function in implementing the class IV md neuron identity. According to our model (Fig. 6), at least one unidentified class IV md neuron-specific TF is required for activating Col expression and regulating the level of Cut (Cut^{medium}) expression. On one side, Col cooperates with other md-specific TFs to activate *ppk* transcription specifically in class IV md neurons, independent of Cut. On the other, Col and Cut^{medium} are involved in establishing the secondary complex dendrite network, typical of these neurons that develops in larvae. Since *col* transcription is not maintained beyond embryogenesis, its role in secondary dendrite branching and maintaining *ppk* transcription in larvae must be indirect and likely involves (an) intermediate TF(s). Systematic identification of *col* and *cut* targets in embryonic class IV md neurons should allow to better understand their respective roles. *col* involvement in two parallel regulatory networks (or dual function) links two salient features of class IV md neurons, an extended dendritic field and *ppk* expression. Dendrites act as information-integrating centres as they receive sensory or synaptic inputs. How expression of the Na+ channel subunit Ppk and extended dendrite arborisation are physiologically linked is a next question.

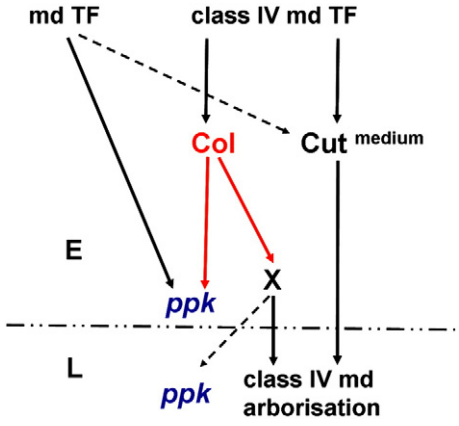


Fig. 6. A model for Col function in class IV md neurons. Transcription factors (TF) responsible for specifying class IV md neurons both activate *col* transcription and control medium levels of Cut. Together with md TF, Col activates *ppk* transcription. Since *col* is only expressed in md neurons during embryogenesis, we postulate that its requirement for the dendrite branching, that takes place in larvae, passes by the activation of unknown TFs (X). Col requirement is independent of Cut. Whether maintenance of *ppk* expression in larval stages requires X remains a possibility (dashed arrow). Arrows indicate a positive control, whether direct or indirect.

Note added in proof

During the submission process of our manuscript, we became aware of a parallel study by Hattori et al., 2007, reporting very similar conclusions. After acceptance, we also became aware of a similar study by Jinushi-Nakao et al., 2007.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.12.030.

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